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Accuracy of Antinuclear Antibody, anti-double stranded DNA Antibody, and Anti-histone Antibody Tests in the Setting of Antiphospholipid Antibodies

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Yale University

1996

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Accuracy of Antinuclear Antibody, anti-double stranded DNA Antibody, and Anti-histone Antibody Tests in the Setting of Antiphospholipid Antibodies

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

DAVID SHIAO-LOONG WANG
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ABSTRACT

ACCURACY OF ANTINUCLEAR ANTIBODY, ANTI-DOUBLE STRANDED DNA ANTIBODY, AND ANTI-HISTONE ANTIBODY TESTS IN THE SETTING OF ANTIPHOSPHOLIPID ANTIBODIES.

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The antiphospholipid syndrome is a recently recognized clinical entity manifested by disease related to sequelae of thrombosis. Antiphospholipid antibodies associated with clinical disease can occur as a primary syndrome or in association with autoimmune diseases, especially systemic lupus erythematosus (SLE). Because serologic laboratory tests are important in the diagnosis and management of patients with SLE, this research intended to assess the reactivity of anticardiolipin antibodies (ACA) with substrates in commercially available lupus-related serologic tests for antinuclear antibody (ANA), double-stranded DNA (dsDNA), and histone antibodies. Sera from the Yale Rheumatology Diagnostic Laboratory were selected that had previously been reported as ACA positive. Sera were characterized for ACA by ELISA, ANA by HEp-2 cell immunofluorescence, anti-dsDNA antibodies by Crithidia immunofluorescence, and anti-histone antibodies by ELISA. ACA were adsorbed from ACA positive sera which also tested positive for ANA, anti-dsDNA, or histone antibodies, after which sera were re-assayed for these antibodies. After all serologic results were obtained, available charts were reviewed to correlate the serologic findings with the clinical presentation and diagnosis of patients whose sera had been analyzed. The

results suggest that the presence of ACA in sera from patients without evidence of SLE can give rise to a false positive anti-dsDNA by Crithidia testing.

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INTRODUCTION

Background

The antiphospholipid antibody syndrome (APS) is a clinical syndrome of recurrent venous and/or arterial thrombotic events that occurs in the setting of two principal types of autoantibodies: the lupus anticoagulant and anticardiolipin antibody (ACA).[23] The syndrome was initially described in 1986[10] in patients with underlying systemic lupus erythematosus (SLE)[8] and therefore was originally thought to comprise a subset of SLE.[26] However, it is now clear that the two can occur independently.[17] The terms primary and secondary APS are used to distinguish between APS that occurs as a single disease entity and APS that arises in patients with other underlying disease processes, especially SLE.[5,7,23]

Clinical Manifestations of APS

Though the manifestations of APS are highly variable, the three principal presentations of patients with APS are arterial and venous thromboses in a young individual, recurrent pregnancy loss, or thrombocytopenia.[1,11,22,23]

Arterial thrombotic events usually occur as discrete episodes involving single vessels. Arterial occlusions have been shown to involve vessels of all sizes, from large vessels such as the aorta to small capillaries. Stroke and other neurological events are perhaps the most striking clinical presentations of arterial occlusion due to APS. In one study, antiphospholipid antibodies were implicated in the pathogenesis of cerebral infarction in up to 7% of stroke patients.[11] Other arterial thrombotic events result in myocardial infarctions, renovascular hypertension, gangrene, and blindness. There have

even been reports of intestinal, hepatic, and adrenal infarction leading to Addison's disease.[16]

Venous thrombosis due to APS can produce equally serious sequelae. Disease manifestations include superficial thrombophlebitis, deep venous thrombosis, pulmonary embolus, Budd-Chiari syndrome, and renal vein thrombosis. A catastrophic occlusion syndrome associated with APS has been suggested by Asherson in which patients are critically ill and resemble those with disseminated intravascular coagulation.[3] Although APS is a less common cause of venous occlusion than other hypercoagulable conditions, it is important to include APS in the differential diagnosis of a patient with otherwise unexplained venous thrombosis.[8,16]

Recurrent fetal loss due to APS is secondary to placental infarction and other placental vascular anomalies associated with thrombosis. APS-induced pregnancy losses most commonly occur after the first trimester. Women with otherwise unexplained second or third trimester fetal loss or multiple recurrent fetal losses should undergo serological screening for antiphospholipid antibodies.[8] As a result of increased recognition of the association between antiphospholipid antibodies and fetal loss, more women have completed successful pregnancies due to earlier diagnosis and interventional therapy.

The final major manifestation of APS, thrombocytopenia, is frequent, but generally not a cause of clinically significant bleeding. These patients most often are diagnosed with APS after investigation of abnormal platelet counts obtained for other medical reasons.[11]

Because the clinical manifestations of APS relate to thrombosis, APS can involve any organ system. Associated features such as livedo reticularis, hemolytic anemia, valvular heart disease with sterile vegetations, transverse

myelitis, pulmonary hypertension, migraine headache, and many others have been reported in patients with APS. Because these clinical features are present in a number of other diseases and the frequency of APS presenting with such manifestations is low, by themselves they rarely are useful clinical indicators of APS.[8,16,22]

Antiphospholipid Antibodies

Antiphospholipid antibodies comprise a group of antibodies that bind either directly to phospholipid or to plasma proteins that bind phospholipid. Antiphospholipid antibodies associated with thrombosis generally belong to the latter subset. Two autoantibodies associated with APS are the lupus anticoagulant and the anticardiolipin antibody (ACA).[23] The lupus anticoagulant is defined by inhibition of the prothrombinase reaction in *in vitro* coagulation tests,[5,28] and in some cases has been shown to be due to antibodies to prothrombin. Laboratory tests for the lupus anticoagulant include the activated partial thromboplastin time, the modified (dilute) Russell viper venom time,[24] and the Kaolin Clotting Time. Prolongation of any of the above *in vitro* tests that is not corrected by adding normal sera (to account for clotting factor deficiencies) defines the presence of the lupus anticoagulant.[23,27]

ACA is the other autoantibody that can be present in patients with APS.[23] These autoantibodies are most commonly detected by ELISA using cardiolipin as a substrate.[2,9] It has recently been shown, however, that the ACAs associated with clotting do not directly bind cardiolipin,[14] but rather bind the plasma protein $ß_2$-glycoprotein I or a $ß_2$-glycoprotein I-phospholipid complex.[15,28] Although only weakly bound by ACA in plasma, immobilization of $ß_2$-glycoprotein I by polystyrene or cardiolipin on ELISA

plates results in markedly enhanced binding of ACA to this protein.[12] ß$_2$-glycoprotein I therefore enhances the binding of autoimmune ACAs to cardiolipin as detected by ELISA.[25] The mechanism by which antibodies to ß$_2$-glycoprotein I induce clotting is unknown, but ß$_2$-glycoprotein I has been shown to inhibit the intrinsic coagulation pathway.[29] Because ß$_2$-glycoprotein I appears to be a necessary cofactor for pathogenic ACA binding, the binding of ACAs associated with infections, e.g. syphilis, in the absence of ß$_2$-glycoprotein I suggests that these infection-induced ACAs bind directly to phospholipid rather than plasma proteins that bind phospholipid.[27,29] Differences in primary binding antigens of ACAs offer one explanation for why infection-induced ACAs are not normally associated with clotting events.

Pathophysiology

Despite the wealth of knowledge concerning APS that has accumulated over the past decade, its pathophysiology and pathogenesis remain a mystery. It is unknown whether antiphospholipid antibodies directly lead to thrombosis or whether they represent a serologic marker for a hypercoagulable state.[18]

Proposed mechanisms of action include the effects of antiphospholipid antibodies on different cells, clotting factors, and other proteins involved in the coagulation cascade. Suggested mechanisms of thrombosis include interference with ß$_2$-glycoprotein I activity, imbalance in the prostacyclin and thromboxane pathways, interference with antithrombin III, protein C, or protein S activity, and many others.[8,18] Clearly, while there exists a strong association between antiphospholipid antibodies and thrombosis, a precise mechanism by which this occurs has not yet been established.

Differentiation between Primary APS and Secondary APS in Association with SLE:

Although originally thought to be a subset of lupus patients, patients can develop APS in the absence of lupus or other autoimmune diseases. There are several autoantibodies seen in SLE that are helpful in confirming the diagnosis of SLE. Antinuclear antibodies (ANA) are an excellent screening test for SLE, with a sensitivity reported as high as 95%.[19] However, a positive ANA is not specific for SLE; ANAs occur in other autoimmune diseases, chronic inflammatory processes, viral infections, and can be induced by several medications; in addition, ANAs occur in some normal individuals.[6,13] Thus, although a useful screening test with a high predictive value if negative, presence of an ANA is not pathognomonic for SLE. Patients with SLE usually have ANAs with multiple specificities. For example, patients may have antibodies to both double-stranded DNA (dsDNA) as well as histones, proteins that bind DNA. The recognition that antibodies bind multiple antigens in a patient supports the diagnosis of an autoimmune disease process such as SLE. Anti-histone antibodies by themselves are not diagnostic of SLE.

In contrast, 30-60% of patients with SLE have antibodies to native or dsDNA. Antibodies to native DNA are only found in SLE patients and are a diagnostic criterion for SLE. Although titers of anti-dsDNA antibodies do not always reflect SLE disease activity and are not necessarily helpful in following the clinical course of all patients with SLE, antibodies to dsDNA have been implicated in the pathogenesis of renal disease in SLE patients, and titers of high avidity anti-dsDNA antibodies correlate with renal disease activity.[6,13,19]

Thus, the presence of anti-dsDNA antibodies carries significant diagnostic and prognostic value. There are several techniques for measuring

antibodies to dsDNA. The most commonly used assays in most diagnostic laboratories are the ELISA anti-dsDNA antibody assay and the Crithidia luciliae immunofluorescence assay. The ELISA anti-dsDNA antibody screening assay involves incubating sera on dsDNA coated plates and measuring quantitative binding of antibodies. The ELISA is the best screening assay, as it has the greatest sensitivity of all anti-dsDNA antibody tests. It measures, however, both low and high avidity anti-dsDNA antibodies as well as antibodies to denatured dsDNA (which may include ssDNA antibodies found also in non-lupus disorders) and therefore is less reliable as a specific marker for SLE or SLE renal disease.[19,30] The Crithidia luciliae immunofluorescence assay is a test that measures anti-dsDNA antibodies in sera through the binding of antibodies to the kinetoplast on the protozoan *Crithidia luciliae,* which is a pure source of dsDNA, free of ssDNA. Although the Crithidia assay is less sensitive for anti-dsDNA antibodies than ELISA, it is a more useful test clinically because of its greater specificity, particularly for high avidity antibodies to dsDNA, which correlate with renal disease in SLE patients. A third technique, the Farr assay, is reputedly the most specific test for pathogenic anti-dsDNA antibodies; however, it is not widely available because it is expensive, employs radioactivity, and is technically more laborious.[30]

CASE PRESENTATION

Recently, a previously healthy 19-year-old white male presented to Yale-New Haven Hospital with a pulmonary embolus and in acute renal failure. Laboratory analysis revealed a strongly positive ACA. Further testing revealed a positive ANA and equivocally positive anti-dsDNA antibodies as measured by ELISA. Based on the findings of positive ANA and the borderline anti-dsDNA antibody test, the patient was given the clinical diagnosis of APS in association with SLE and probable lupus nephritis.

To clarify laboratory tests, a rheumatology consultant suggested repeating the anti-dsDNA antibody tests, using the Crithidia luciliae immunofluorescence assay. Surprisingly, the Crithidia test, which is a less sensitive but more specific assay for anti-dsDNA antibody detection than the ELISA, was strongly positive, whereas an ELISA performed on the same sera was negative. Because the ACA was very high titer, it was postulated that the presence of ACA might contribute to the reactivity detected to dsDNA, yielding a false positive anti-dsDNA antibody by Crithidia.

The ACA from this patient's sera was removed by affinity adsorption. Adsorption of ACA from the sera resulted in a loss of reactivity for dsDNA by all anti-dsDNA antibody tests on repeat testing, whereas a control serum from an ACA negative patient with known lupus nephritis and anti-dsDNA antibodies did not lose reactivity by Crithidia or ELISA after similar treatment. This supported the notion that the patient's ACA might have contributed to a false positive anti-dsDNA antibody test result.

As noted above, histone antibodies frequently accompany anti-dsDNA antibodies in patients with SLE. Histones contain a charged region homologous to the phospholipid binding site on $ß_2$-glycoprotein I, a target of some ACAs. It was thus postulated that the high-titer ACA might also

influence histone antibody testing, although in this patient the anti-histone antibody test was negative.

Review of the present medical literature revealed no information regarding the specificity of tests for ANA, anti-dsDNA antibodies, and anti-histone antibodies in the presence of ACA. The fact that this patient's anti-dsDNA antibody activity by Crithidia and ELISA disappeared following adsorption of ACA from his sera suggested that high-titer ACAs might lead to false positive anti-dsDNA and other autoantibody testing in ACA positive patient sera. The implications of this finding could be clinically significant and impact on patient diagnosis and management, especially considering that the presence of anti-dsDNA antibodies by Crithidia is regarded as a very specific laboratory finding supporting SLE as well as a marker associated with lupus nephritis.

HYPOTHESIS

Based on the case described, it is hypothesized that the presence of high-titer ACA could lead to false positive anti-dsDNA and other autoantibody testing important for the diagnosis of autoimmune diseases such as SLE.

PURPOSE OF STUDY

The purpose of this study was to determine whether the presence of ACA in sera affects standard commercially available laboratory tests for SLE: ANA by HEp-2 cell immunofluorescence, anti-dsDNA antibody by ELISA and Crithidia, and anti-histone antibody tests by ELISA.

OVERALL EXPERIMENTAL DESIGN

Frozen sera specimens from the Yale Rheumatology Diagnostic Laboratory Sera Bank previously reported as positive for ACA were thawed and retested for ACA reactivity. Those sera that were ACA positive on retesting were further evaluated for ANA, anti-dsDNA antibody, and anti-histone antibody tests both before and after adsorption of ACA from the sera. Results were then statistically analyzed and conclusions were drawn regarding specificity of ANA, anti-dsDNA, and histone antibody tests in the presence of ACA. Upon completion of the study, historical features of the patient population were obtained by chart review to correlate with experimental findings.

MATERIALS AND METHODS

All methods described here were performed by the author.

Selection of Patient sera:

Sera from sixty-six patients were used for this study. Sera were selected by reviewing sample test results for ACA, ANA, and anti-dsDNA antibody from the Yale Rheumatology Diagnostic Laboratory Sera Bank over the past six years. Sera were selected from patients who had all been previously identified as having positive ACA in a standard ELISA assay. Sera included in the study were selected based on the presence of high-titer ACA IgG (>2 times control) and/or the presence of concomitant positive ANA and anti-dsDNA antibody in addition to positive ACA. Sera were obtained in a fashion blinded to the underlying diagnosis or clinical features of the patients. Sera were at the onset aliquoted in triplicate to avoid the effects of repeated

freeze/thaw cycles on antibodies. Sera had been stored at -20°C for up to six years.

Anticardiolipin Antibody Assay:

Cardiolipin antigen (Sigma Chemical Company, St. Louis, MO) was diluted 116 µl in 10.4 ml of absolute ethanol, and 50 µl was aliquoted to each well of a Titertek 96 well flat plate (Flow Labs). Plates were kept uncovered at 4°C overnight to allow for evaporation of ethanol reagent. Plates were washed three times with PBS with 0.02% azide (Sigma Chemical Company, St. Louis, MO) using an automated plate washer, then blocked with PBS and 10% fetal calf serum (Gibco) with 0.02% azide for one hour at 20°C, covered. Plates were again washed three times as above, and 50 µl of 1:50 dilution patient sera or control sera was added to each well. Control sera included recently tested ACA positive sera as a positive control, normal human sera as a negative control, and Harris Standards (Louisville APL Diagnostics, Inc., Louisville, AL). Plates were incubated for two hours at 20°C, covered to prevent evaporation. Plates were washed as above, 50 µl of 1:1000 dilution goat anti-human IgG (λ heavy chain specific) alkaline phosphatase labeled IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) was added to each well, and plates were incubated two hours at 20°C, covered. Plates were washed as above and 50 µl of pNPP substrate (Sigma Chemical Company, St. Louis, MO) prepared according to pNPP kit instructions supplied by the manufacturer was added to each well. Plates were incubated covered in the dark at 20°C for 15 minutes (or until positive control IgG was at least 1.000 OD) and read immediately on a Titertek Multiskan plate reader fitted with a 405 nm filter. A standard reference curve was constructed using the Harris Standard reference controls, and the ACA reactivity of each sample was

reported in GPL units by comparing absorbance values against the standard curve.

Adsorption of Anticardiolipin Antibodies from Sera:

Cardiolipin-coated ELISA plates were prepared as described above. The first row of twelve wells (Row A on 96 well flat plates) was washed three times with PBS with azide, and 100 µl of patient sera diluted 1:10 in PBS was added to the first row of twelve wells along with negative controls. Plates were incubated for 30 minutes at 20°C, covered. The second row of wells (Row B on 96 well flat plates) was washed three times as above, and the diluted sera from the first row (Row A) was carefully transferred to the second row (Row B) using a multi-channel Titertek pipette taking caution to lose as little sera as possible between transfers. Following the transfer, 100 µl of blocking solution (10% FCS in PBS with 0.02% azide) was replaced into each well of the first row (Row A), and the plate was allowed to incubate for 30 minutes. This procedure was repeated until the diluted patient sera was transferred to the eighth row of twelve wells (Row G) and the remaining wells contained blocking solution. The diluted patient sera was then transferred to another fresh pre-blocked cardiolipin coated plate and the adsorption procedure was repeated on the second plate. ELISA was performed on the plates from which the sera had been transferred in order to determine if ACA had been fully adsorbed out of patient sera by comparing with negative controls. Those patient sera which were still positive for ACA following adsorption from two plates were transferred to a third 96 well plate. Sera was transferred throughout the entire width of the third plate, and ELISA was performed on the third plate.

Antinuclear Antibody Assay (HEp-2 Cell Immunofluorescence):

Antinuclear antibody indirect immunofluorescence assays were manufactured by Quidel from San Diego, California, USA. Tests were performed exactly as outlined in the manufacturer's instruction manual, with the exception that Evans blue counterstain was not used. Sera were initially diluted 1:40 and 1:160 in PBS. Further sample dilution was performed as needed. Forty µl of diluted sera was added to each well, along with two positive controls (homogeneous and speckled) and a negative control, all included in the kit. Slides were incubated in a humidified chamber for 20 minutes, rinsed briefly with a stream of PBS, and washed in PBS for 10 minutes at room temperature. Slides were then overlaid with fluorescein-conjugated goat anti-human IgG and incubated in a humidified chamber for 20 minutes in the dark. Upon removal, slides were again washed as outlined above. Cover slips were then applied over a few drops of mounting media supplied by the kit. Lastly, slides were viewed using a Zeiss immunofluorescent microscope.

Crithidia luciliae Immunofluorescence Assay:

Crithidia luciliae anti-dsDNA assays were performed using CrithiDNA Anti-nDNA Antibody test kits manufactured by Antibodies Incorporated from Davis, California, USA. Tests were performed exactly as outlined in the manufacturer's instruction manual. Sera were diluted 1:10 in IFA-PBS (supplied by the kit) and ten µl of diluted test sera were added to each well on the substrate slides, along with both positive and negative controls included in the kit. Slides were incubated in a moist chamber for 30 minutes, rinsed briefly with a stream of IFA-PBS, washed for 10 minutes in IFA-PBS, and dipped in distilled water. Slides were then overlaid with fluorescent labeled

goat anti-human immunoglobulin and incubated for 30 minutes in the dark and washed again as outlined above. Cover slips were then applied over a few drops of buffered glycerol mounting media. Lastly, slides were viewed using a Zeiss immunofluorescent microscope.

Anti-dsDNA ELISA:

Anti-dsDNA ELISA kits were manufactured by Apotex Scientific, Incorporated, from Arlington, Texas, USA. Tests were performed exactly as outlined in the manufacturer's instruction manual. Sera were diluted 1:10 in Apotex sample diluent. One hundred µl of each prediluted sample sera or 100 µl of Apotex anti-dsDNA reference controls were added to the appropriate wells. The plates with samples and controls were incubated for 60 minutes at room temperature. After decanting the contents of the plate, each well was washed three times with 200 µl of Apotex wash buffer per well, following which 100 µl of Apotex dsDNA conjugate was added to each well. The plates were allowed to incubate for 30 minutes at room temperature. The contents of the plate were decanted, and the plates were washed three times as described above, and 100 µl of Apotex substrate was added to each well. After another 30 minute incubation, 100 µl of Apotex stop solution was added to each well. The plates were immediately read on a Titertek Multiskan plate reader fitted with a 550 nm filter. A standard reference curve was constructed using the reference controls, and the anti-dsDNA antibody reactivity of each sample was reported in IU/ml by comparing absorbance values against the standard curve.

Anti-histone ELISA:

Anti-histone ELISA kits were manufactured by Apotex Scientific, Incorporated, from Arlington, Texas, USA. Tests were performed exactly as outlined in the manufacturer's instruction manual. Sera were initially diluted 1:10 in Apotex sample diluent. One hundred µl of each prediluted sample sera or 100 µl of Apotex anti-histone reference controls were added to the appropriate wells. The plates with samples and controls were incubated for 60 minutes at room temperature. After decanting the contents of the plate, each well was washed three times with 200 µl of Apotex wash buffer per well, following which 100 µl of Apotex histone conjugate was added to each well. The plates were allowed to incubate for 30 minutes at room temperature. The contents of the plate were decanted, and the plates were washed three times as described above, and 100 µl of Apotex substrate was added to each well. After another 30 minute incubation, 100 µl of Apotex stop solution was added to each well. The plates were immediately read on a Titertek Multiskan plate reader fitted with a 550 nm filter. A standard reference curve was constructed using the reference controls, and the histone reactivity of each sample was reported in IU/ml by comparing absorbance values against the standard curve.

Review of Patient Charts:

Following the completion of all laboratory experiments, available patient charts in the Department of Medical Records at Yale-New Haven Hospital were reviewed. Attention was given to the discharge summaries to review clinical features and discharge diagnoses to determine whether patients had other disease states associated with their positive ACA. In addition, charts were reviewed to search for the reason that the ACA tests had

been ordered, e.g. to evaluate unexplained thrombosis or recurrent pregnancy loss.

Statistical Analysis:

Statistical analysis of data was performed after completion of all laboratory experiments. Statistical analysis was performed after discussion with James H. Jekel, M.D., M.P.H., Professor of Public Health, from the Yale University School of Medicine.

RESULTS

Selection of Sera

Sixty-six sera were selected from patients who had previously tested positive for ACA by ELISA in the Yale Rheumatology Diagnostic Laboratory. Table 1 shows the original characteristics of the initial sample sera selected for the project.

Table 1-Initial Sample Sera Characteristics

Spec #	Reported ACA IgG	Orig Ctrl	IgG/Ctl Ratio	ANA (If avail)	ANA Tit (If avail)	AntiDNA (If avail)	DNA Tit (If avail)
1	399	37	10.78				
2	636	47	13.53				
3	338	47	7.19	Positive		Negative	
4	1037	40	25.93	Negative		Negative	
5	769	45	17.09	Positive		Negative	
6	362	121	2.99	Positive	1:1280	Negative	
7	372	68	5.47				
8	579	61	9.49				
9	624	96	6.5	Positive	1:160	Positive	
10	635	72	8.82	Positive	1:2560	Positive	
11	336	58	5.79	Positive	1:320	Positive	1:80
12	363	66	5.5				
13	428	81	5.28				
14	605	45	13.44				
15	194	69	2.81	Positive	1:640	Positive	1:10
16	66	61	1.08	Positive	1:81920	Positive	1:1280
17	672	73	9.21	Positive	1:640	Positive	
18	804	289	2.78	Positive	1:160	Negative	
19	288	137	2.1	Positive	1:5120	Positive	
20	436	149	2.93	Negative		Negative	
21	719	149	4.83	Negative		Negative	
22	633	100	6.33	Positive	1:40	Negative	
23	432	154	2.81				
24	422	98	4.31				
25	1046	84	12.45	Positive	1:640	Negative	
26	837	82	10.21				
27	575	82	7.01				
28	325	82	3.96	Negative		Negative	
29	786	108	7.28				
30	441	136	3.24	Negative		Negative	
31	407	73	5.58				
32	896	135	6.64	Negative		Negative	
33	519	102	5.09	Positive		Negative	
34	419	105	3.99	Negative		Negative	
35	399	154	2.59				
36	933	154	6.06	Negative		Negative	
37	338	117	2.89				
38	610	210	2.9				
39	415	188	2.21				
40	407	109	3.73				
41	452	109	4.15	Positive	1:2560	Positive	
42	530	102	5.2				
43	348	102	3.41				
44	659	138	4.78				

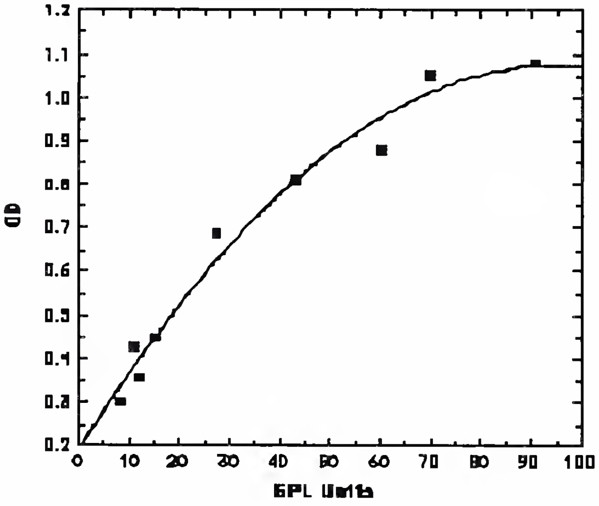
Table 1-Initial Sample Sera Characteristics

Spec #	Reported ACA IgG	Orig Ctrl	IgG/Ctl Ratio	ANA (If avail)	ANA Tit (If avail)	AntiDNA (If avail)	DNA Tit (If avail)
45	375	60	6.25	Negative		Negative	
46	175	130	1.35	Positive	1:1280	Positive	1:320
47	388	166	2.34				
48	484	155	3.12				
49	642	106	6.06	Positive	1:20480	Negative	
50	1005	141	7.13	Positive	1:320	Negative	
51	491	213	2.31				
52	117	100	1.17	Positive	1:160	Positive	
53	532	350	1.52				
54	372	235	1.58	Positive	1:1280	Negative	
55	515	405	1.27	Positive	1:10240	Positive	1:40
56	492	405	1.21	Positive	1:5120	Positive	1:320
57	564	125	4.51	Positive	1:5120	Positive	1:160
58	1680	233	7.21	Positive	1:10240	Positive	1:160
59	796	104	7.65	Positive	1:1280	Negative	
60	799	172	4.65	Positive	1:40	Negative	
61	871	276	3.16	Positive	1:40	Negative	
62	728	97	7.51	Positive	1:320	Negative	
63	449	294	1.53	Negative		Negative	
64	333	150	2.22				
65	404	345	1.17	Positive	1:1280	Positive	1:80
66	419	330	1.27				

Anticardiolipin Antibody Assay

Of the 66 sera tested for ACA at the beginning of the project, 58 were ACA positive (87.9%) and 8 were ACA negative (12.1%) on repeat testing. In order to assure that the originally reported ACA results were not different from the results obtained in this experiment, the reported results were compared with the experiment results by comparing the ACA IgG/Control IgG ratio. By using a standard paired t-test on the ACA IgG/Control ratios, the differences in the ACA IgG/Control ratio in the reported ACA and the experiment results were not statistically significant ($p>0.5$). Table 2 gives a comparison of the reported ACA and the experimental ACA results. Values obtained for ACA in the current study were reported in GPL units as determined by plotting individual results against a standard curve generated from the Harris Standard reference controls. Prior to last year, ACA results from the Yale Rheumatology Diagnostic Laboratory were not reported in GPL units, so a comparison based on Harris Standard GPL units could not be made. A representative graph of the standard curve typical of those used in the ELISA ACA assays is shown below:

Standard ACA Curve Using Harris Standards



GPL Units:

>80	High Positive
20-80	Moderate
<20	Low Positive
<10	Cut-Off

Table 2-Reported ACA vs. Pre-Adsorption ACA

Spec #	Reported ACA IgG	Rep Ctrl	IgG/Ctl Ratio	Pre-Ads ACA IgG	Pre-Ads Ctrl	IgG/Ctl Ratio	GPL Units
1	399	37	10.78	1026	181	5.67	75
2	636	47	13.53	978	181	5.4	68
3	338	47	7.19	945	181	5.22	58
4	1037	40	25.93	1149	181	6.35	>80
5	769	45	17.09	879	181	4.86	50
6	362	121	2.99	235	181	1.3	3
7	372	68	5.47	1147	181	6.34	>80
8	579	61	9.49	460	181	2.54	18
9	624	96	6.5	610	161	3.79	26
10	635	72	8.82	795	161	4.94	43
11	336	58	5.79	890	161	5.53	55
12	363	66	5.5	880	161	5.53	53
13	428	81	5.28	906	161	5.63	56
14	605	45	13.44	867	161	5.39	52
15	194	69	2.81	794	161	4.93	43
16	66	61	1.08	226	161	1.4	3
17	672	73	9.21	1145	161	7.11	>100
18	804	289	2.78	1089	161	6.76	>100
19	288	137	2.1	372	161	2.31	12
20	436	149	2.93	857	161	5.32	50
21	719	149	4.83	930	161	5.78	61
22	633	100	6.33	932	161	5.79	61
23	432	154	2.81	1056	161	6.56	93
24	422	98	4.31	918	152	6.04	62
25	1046	84	12.45	1007	152	6.63	>100
26	837	82	10.21	970	152	6.38	85
27	575	82	7.01	131	152	0.86	0
28	325	82	3.96	504	152	3.32	18
29	786	108	7.28	747	152	4.91	38
30	441	136	3.24	360	152	2.37	11
31	407	73	5.58	996	152	6.55	93
32	896	135	6.64	871	152	5.73	55
33	519	102	5.09	853	152	5.61	51
34	419	105	3.99	245	152	1.61	5
35	399	154	2.59	198	152	1.3	2
36	933	154	6.06	897	152	5.9	58
37	338	117	2.89	598	152	3.93	26
38	610	210	2.9	805	152	5.3	45
39	415	188	2.21	371	166	2.23	7
40	407	109	3.73	1099	166	6.62	75
41	452	109	4.15	1049	166	6.32	57
42	530	102	5.2	1303	166	7.85	>100
43	348	102	3.41	678	166	4.08	24
44	659	138	4.78	1192	166	7.18	>100

Table 2-Reported ACA vs. Pre-Adsorption ACA

Spec #	Reported ACA IgG	Rep Ctrl	IgG/Ctl Ratio	Pre-Ads ACA IgG	Pre-Ads Ctrl	IgG/Ctl Ratio	GPL Units
45	375	60	6.25	1263	166	7.61	>100
46	175	130	1.35	501	166	3.02	12
47	388	166	2.34	838	166	5.05	33
48	484	155	3.12	1137	166	6.85	>100
49	642	106	6.06	1117	166	6.73	>100
50	1005	141	7.13	1202	166	7.24	>100
51	491	213	2.31	620	166	3.73	18
52	117	100	1.17	371	166	2.23	7
53	532	350	1.52	706	166	4.26	24
54	372	235	1.58	717	146	4.91	35
55	515	405	1.27	298	146	2.04	7
56	492	405	1.21	434	146	2.97	13
57	564	125	4.51	1083	146	7.72	92
58	1680	233	7.21	1192	146	8.16	>100
59	796	104	7.65	1205	146	8.25	>100
60	799	172	4.65	1048	146	7.18	77
61	871	276	3.16	1232	146	8.44	>100
62	728	97	7.51	1145	146	7.84	>100
63	449	294	1.53	660	146	4.52	32
64	333	150	2.22	838	146	5.74	48
65	404	345	1.17	365	146	2.5	10
66	419	330	1.27	511	146	3.5	19

ANA, Anti-dsDNA, and Anti-Histone Results before ACA Adsorption

Of the 66 sera tested for ANA by HEp-2 cell immunofluorescence, 45 were ANA positive (68.2%) and 21 were ANA negative (31.8%).

Of the 66 sera tested for reactivity to dsDNA, 12 tested were anti-dsDNA antibody positive (18.2%) and 54 were negative (81.8%) by Crithidia luciliae immunofluorescence.

Of the 66 sera tested for anti-histone antibodies by ELISA, 25 were anti-histone antibody positive (37.9%) and 41 were negative (62.1%). Results were reported in anti-histone units/ml as determined by plotting individual results against a standard curve. The standard curve used for the anti-histone ELISA assays is shown below:

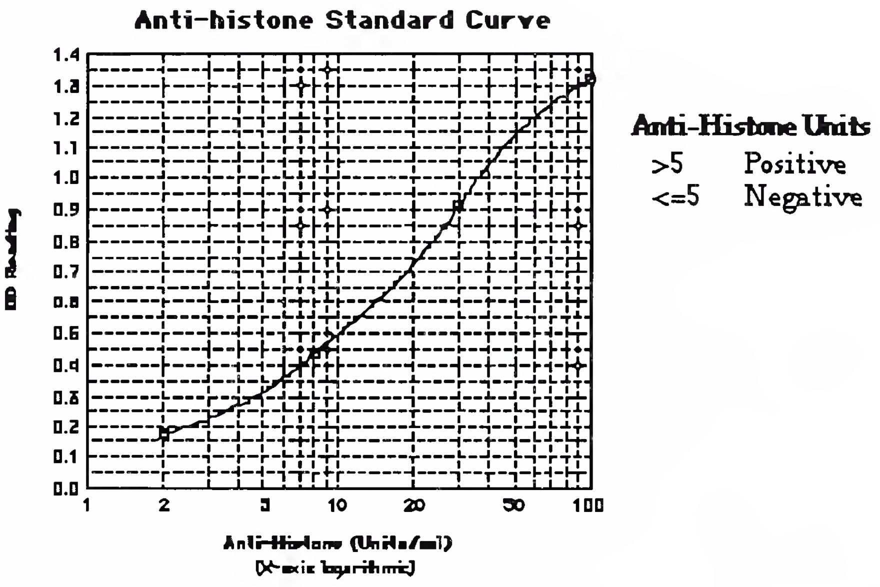


Table 3 gives a summary of ACA, ANA, anti-dsDNA, and anti-histone results of patient sera prior to ACA adsorption.

Table 3-ACA, ANA, Anti-dsDNA, and Anti-histone before Adsorption

Spec #	ACA	GPL	ANA	Patrn	Crithidia	Histone	U/ml
1	Positive	75	Positive	nuc	Negative	Positive	8
2	Positive	68	Positive	speck	Negative	Negative	<2
3	Positive	58	Positive	speck	Negative	Negative	4
4	Positive	>80	Negative		Negative	Positive	21
5	Positive	50	Positive	speck,nuc	Negative	Negative	<2
6	Negative	3	Positive	speck,nuc	Negative	Negative	3
7	Positive	>80	Negative		Negative	Positive	6
8	Positive	18	Negative		Negative	Positive	7
9	Positive	26	Positive	hom	Negative	Negative	2
10	Positive	43	Positive	nuc	Negative	Negative	4
11	Positive	55	Positive	hom	Negative	Negative	3
12	Positive	53	Negative		Positive	Negative	3.5
13	Positive	56	Wk pos	speck	Negative	Negative	4
14	Positive	52	Positive	hom	Negative	Negative	3
15	Positive	43	Positive	hom	Negative	Positive	8
16	Negative	3	Positive	hom	Positive	Positive	>100
17	Positive	>100	Positive	speck	Positive	Positive	12
18	Positive	>100	Positive	hom,nuc	Negative	Negative	3
19	Positive	12	Positive	speck	Negative	Positive	8
20	Positive	50	Negative		Negative	Positive	7
21	Positive	61	Wk pos	speck	Negative	Negative	5
22	Positive	61	Wk pos	speck	Negative	Positive	6
23	Positive	93	Negative		Positive	Negative	4
24	Positive	62	Positive	speck	Negative	Negative	5
25	Positive	>100	Wk pos	speck	Negative	Negative	3
26	Positive	85	Negative		Negative	Positive	6
27	Negative	0	Positive	hom	Negative	Negative	4
28	Positive	18	Negative		Negative	Positive	>100
29	Positive	38	Negative		Negative	Negative	<2
30	Positive	11	Negative		Negative	Negative	4
31	Positive	93	Positive	speck	Negative	Negative	3
32	Positive	55	Negative		Positive	Negative	2
33	Positive	51	Wk pos	speck	Positive	Negative	3
34	Negative	5	Negative		Negative	Negative	<2
35	Negative	2	Negative		Negative	Negative	2
36	Positive	58	Positive	hom	Negative	Negative	<2
37	Positive	26	Positive	speck	Positive	Positive	25
38	Positive	45	Positive	speck	Negative	Positive	8
39	Negative	7	Negative		Negative	Negative	5
40	Positive	75	Negative		Negative	Negative	4
41	Positive	57	Positive	hom	Negative	Positive	6
42	Positive	>100	Positive	hom	Negative	Negative	<2
43	Positive	24	Negative		Negative	Negative	4
44	Positive	>100	Negative		Negative	Negative	<2
45	Positive	>100	Negative		Negative	Negative	2

Table 3-ACA, ANA, Anti-dsDNA, and Anti-histone before Adsorption

Spec #	ACA	GPL	ANA	Patrn	Crithidia	Histone	U/ml
46	Positive	12	Positive	speck	Positive	Positive	21
47	Positive	33	Negative		Negative	Negative	2
48	Positive	>100	Positive	hom	Negative	Positive	8
49	Positive	>100	Positive	hom	Negative	Negative	4
50	Positive	>100	Positive	speck	Negative	Positive	19
51	Positive	18	Wk pos	nuc	Negative	Positive	20
52	Negative	7	Wk pos	speck	Negative	Negative	2
53	Positive	24	Wk pos	speck	Negative	Negative	2
54	Positive	35	Positive	speck,nuc	Negative	Negative	4
55	Negative	7	Positive	speck	Positive	Positive	6
56	Positive	13	Positive	rim	Positive	Positive	60
57	Positive	92	Positive	speck	Positive	Positive	9
58	Positive	>100	Positive	speck	Negative	Positive	39
59	Positive	>100	Negative		Negative	Negative	3
60	Positive	77	Wk pos	speck	Negative	Negative	2
61	Positive	>100	Positive	speck	Negative	Negative	2
62	Positive	>100	Wk pos	speck	Negative	Negative	4
63	Positive	32	Negative		Negative	Negative	<2
64	Positive	48	Positive	hom	Negative	Positive	9
65	Positive	10	Positive	nuc	Positive	Positive	11
66	Positive	19	Wk pos	speck	Negative	Negative	<2

Selection of Sera for ACA Adsorption

After ANA, anti-dsDNA, and anti-histone assays were performed on sera, patient sera that had tested positive for ANA, anti-dsDNA, or anti-histone were selected for ACA adsorption. Sera that had tested negative for ACA by ELISA were not used for further study.

Of the 66 patient sera initially selected by logbook review, 8 sera were negative for ACA on repeat study (Table 3: patients 6, 16, 27, 34, 35, 39, 52, and 55) and an additional 9 sera that tested ACA positive were negative for ANA, anti-dsDNA, and anti-histone antibodies (Table 3: patients 29, 30, 40, 43, 44, 45, 47, 59, and 63). Thus, 17 sera were excluded from analysis and a total of 49 sera were available for further analysis.

Adsorption of ACA was performed on the 49 sera by the method described above. In addition, ACA adsorption was performed on five control sera which were ANA positive, anti-dsDNA antibody positive, but ACA negative. After repetitively transferring sera across two plates, 38 sera were negative for ACA by ELISA testing, and 11 sera were still positive for ACA. The 11 ACA positive sera were transferred to a fresh third plate and ACA adsorption was again performed to attempt loss of reactivity for ACA. Following completion of transfer on a third plate, 8 sera were ACA negative, and 3 sera were still ACA positive. The 3 ACA positive sera were excluded from further study. Further testing at this point was performed on the 46 remaining ACA negative sera.

Post ACA Adsorption Studies

Antinuclear Antibodies:

Forty of the 46 sera used for further analysis originally tested positive for ANA prior to ACA adsorption. ANA assays were performed on the 40

post adsorption sera previously positive for ANA. Five post adsorption sera that had previously tested negative to ANA were used as controls. Of the 40 sera tested, 35 remained ANA positive (88%) and 5 became ANA negative (12%). In 4 out of 5 of the ANA negative sera, the ANA pattern prior to adsorption was speckled, though of extremely low-titer ANA at 1:40. Using McNemar's Chi Square Test with continuity correction, the differences in ANA reactivity following ACA adsorption were not statistically significant with $0.5<p<0.10$. In some sera, however, ANA titer decreased and pattern changed, generally from speckled to homogeneous. Table 4 gives a comparison of ANA activity before and after ACA adsorption.

Table 4-Comparison of ANA Before and After ACA Adsorption

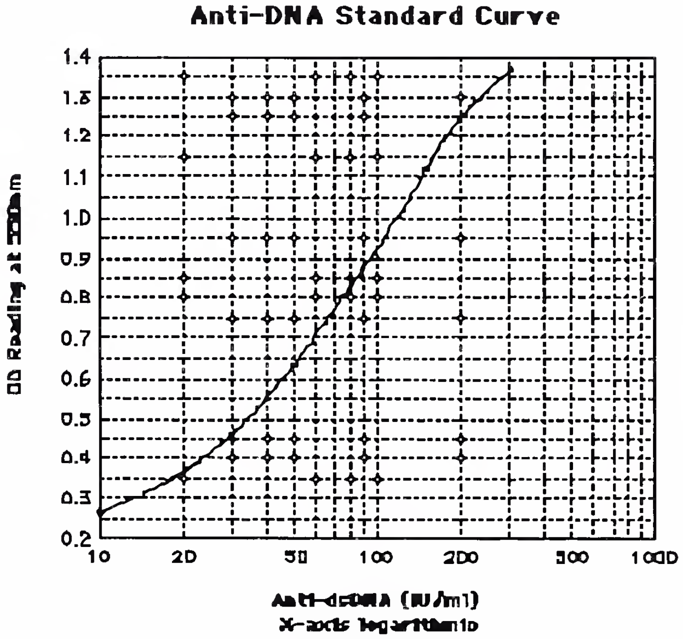
Spec	Prior to ACA Adsorption			After ACA Adsorption			Clinical Diagnoses
#	GPL	ANA	Pattern	ACA	ANA	Pattern	(If Available)
1	75	Positive	nuc	Neg	Wk pos	hom	
2	68	Positive	speck	Neg	Wk pos	speck	
3	58	Positive	speck	Neg	Wk pos	speck	SLE,CVA,CAD,MI
5	50	Positive	speck,nuc	Neg	Positive	speck,nuc	
9	26	Positive	hom	Neg	Wk pos	hom	
10	43	Positive	nuc	Neg	Wk pos	hom	
11	55	Positive	hom	Neg	Positive	hom	
13	56	Wk pos	speck	Neg	Wk pos	speck	
14	52	Positive	hom	Neg	Positive	hom	
15	43	Positive	hom	Neg	Wk pos	hom	
17	>100	Positive	speck	Neg	Positive	hom	
18	>100	Positive	hom,nuc	Neg	Wk pos	hom,nuc	
19	12	Positive	speck	Neg	Wk pos	speck	Preeclampsia,CVA
21	61	Wk pos	speck	Neg	Wk pos	speck	
22	61	Wk pos	speck	Neg	Negative		
24	62	Positive	speck	Neg	Positive	speck	
25	>100	Wk pos	speck	Neg	Negative		APS, Hyperthyroid
31	93	Positive	speck	Neg	Positive	speck	
33	51	Wk pos	speck	Neg	Negative		
36	58	Positive	hom	Neg	Positive	hom	
37	26	Positive	speck	Neg	Positive	speck	
38	45	Positive	speck	Neg	Positive	speck	
41	57	Positive	hom	Neg	Positive	hom	
42	>100	Positive	hom	Neg	Negative		
46	12	Positive	speck	Neg	Wk pos	speck	SLE
48	>100	Positive	hom	Neg	Positive	hom	
49	>100	Positive	hom	Neg	Wk pos	hom	
50	>100	Positive	speck	Neg	Wk pos	hom	SLE,ITP
51	18	Wk pos	nuc	Neg	Wk pos	nuc	SLE,CAD,HTN,Diabetes
53	24	Wk pos	speck	Neg	Negative		
54	35	Positive	speck,nuc	Neg	Positive	speck,nuc	
56	13	Positive	rim	Neg	Positive	hom	SLE,ITP,Autoimm Hemol
57	92	Positive	speck	Neg	Positive	hom	SLE,APS
58	>100	Positive	speck	Neg	Positive	speck	
60	77	Wk pos	speck	Neg	Wk pos	speck	
61	>100	Positive	speck	Neg	Wk pos	speck	
62	>100	Wk pos	speck	Neg	Wk pos	hom	
64	48	Positive	hom	Neg	Wk pos	hom	Asthma, PE, Hemolysis
65	10	Positive	nuc	Neg	Positive	nuc	SLE,Myelitis,Renal Dis
66	19	Wk pos	speck	Neg	Wk pos	speck	Infertility, Ectopic Preg

Abbreviations: nuc=nucleolar;speck=speckled;hom=homogeneous;CVA=Cerebrovascular Accident; CAD=Coronary Artery Disease;MI=Myocardial Infarction;Wk pos=Weak Positive;Neg=Negative

Anti-dsDNA Antibodies:

Ten of the 46 sera used for further study originally tested positive for anti-dsDNA antibodies prior to ACA adsorption. Crithidia anti-dsDNA studies were performed on the ten post adsorption ACA sera. Five post adsorption sera that had previously tested anti-dsDNA antibody negative were used as controls. Of the 10 sera, 6 remained anti-dsDNA antibody positive (60%) and 4 became negative (40%). Using Fisher's exact test, the differences in anti-dsDNA antibody reactivity before and after ACA adsorption were statistically significant with p=0.042.

In addition, anti-dsDNA antibody testing was performed on the 10 sera using ELISA. Five sera were anti-dsDNA antibody positive (50%) and five sera were negative (50%) by ELISA testing. Statistical analysis was not possible on the post adsorption anti-dsDNA ELISA because anti-dsDNA ELISA was not performed prior to ACA adsorption. Table 5 gives a comparison of anti-dsDNA antibody activity before and after ACA adsorption. The standard curve used for the anti-dsDNA antibody ELISA assay is shown below:



Anti-DNA Units:

<30	Negative
30-50	Borderline
50-300	Positive
>300	Strong Positive

Table 5-Comparison of anti-dsDNA Antibodies Before And After ACA Adsorption

Spec	Before ACA Adsorption			After ACA Adsorption					Clinical Diagnoses
#	GPL	Crithidia	Titer	ACA	Crithidia	Titer	ELISA	Int U	(If Chart Available)
12	53	Positive	1:40	Neg	Negative		Negative	26	HIV, CAD, Diabetes Mellitis, TIA, Hypertension
17	>100	Positive	1:160	Neg	Positive	1:160	Positive	120	Chart Not Available
23	93	Positive	>1:320	Neg	Negative		Negative	20	HIV
32	55	Positive	1:160	Neg	Negative		Negative	11	APS, h/o Pregnancy Loss
33	51	Positive	1:80	Neg	Negative		Negative	23	Chart Not Available
37	26	Positive	>1:320	Neg	Positive	1:80	Positive	80	Chart Not Available
46	12	Positive	>1:320	Neg	Positive	>1:320	Positive	210	SLE
56	13	Positive	>1:320	Neg	Positive	>1:320	Positive	>300	SLE, Autoimmune Hemolytic Anemia, Interstitial Lung Disease
57	92	Positive	1:160	Neg	Positive	1:40	Negative	26	SLE, APS
65	10	Positive	>1:320	Neg	Positive	>1:320	Positive	>300	SLE,Myelitis,Renal Disease

Abbreviations: HIV=Human Immunodeficiency Virus;CAD=Coronary Artery Disease;TIA=Transient Ischemic Attack

Anti-histone Antibodies:

Twenty-three of the 46 sera used for further analysis originally tested positive for anti-histone antibodies. Due to limited resources, 19 of the 23 sera were selected in a random fashion and were subsequently tested for antibodies to histone by ELISA.

The 19 sera had all previously tested positive to anti-histone antibodies prior to adsorption. Of 19 sera tested, 15 remained anti-histone positive (79%), and 4 became anti-histone negative (21%). Results for anti-histone reactivity were reported in anti-histone units/ml. By using a standard two-tailed paired t-test, the differences in anti-histone reactivity before and after ACA adsorption were not statistically significant ($0.5<p<0.10$). Table 6 gives a comparison of anti-histone activity before and after ACA adsorption.

Table 6-Comparison of AntiHistone Antibodies Before And After ACA Adsorption

Spec #	Prior to ACA Adsorption			After ACA Adsorption			Clinical Diagnoses
	GPL	Histone	Units/ml	ACA	Histone	Units/ml	(If Available)
4	>80	Positive	21	Neg	Positive	6	CVA,SLE
7	>80	Positive	6	Neg	Negative	4	CVA,IBD
8	18	Positive	7	Neg	Positive	9	AIDS,CMV,PAN,HEP
15	43	Positive	8	Neg	Positive	5	Preeclampsia,CVA
17	>100	Positive	12	Neg	Positive	11	
19	12	Positive	8	Neg	Negative	4	
20	50	Positive	7	Neg	Negative	3	PIH,IUGR
28	18	Positive	>100	Neg	Positive	20	
37	26	Positive	25	Neg	Positive	35	
38	45	Positive	8	Neg	Positive	6	
46	12	Positive	21	Neg	Positive	19	SLE
48	>100	Positive	8	Neg	Positive	7	
50	>100	Positive	19	Neg	Positive	6	SLE,Idiopathic Thromb Purp
51	18	Positive	20	Neg	Positive	9	SLE,CAD,DM,HTN
56	13	Positive	60	Neg	Positive	50	SLE,Autoimmun Hemolysis,ITP
57	92	Positive	9	Neg	Negative	3	SLE,APS
58	>100	Positive	39	Neg	Positive	25	
64	48	Positive	9	Neg	Positive	9	Asthma,Hemolytic Anemia,PE
65	10	Positive	11	Neg	Positive	12	SLE,Myelitis,Renal Disease

bbreviations:Neg=Negative;CVA=Cerebrovascular Accident;IBD=Inflamm Bowel Dis;PAN=Pancreatitis;HEP=Hepatitis;PIH=Pregn
duced Hypertension;CAD=Coronary Artery Disease;DM=Diabetes;HTN=Hypertension;PE=Pulmonary Embolus

Review of Patient Charts

Following the completion of all laboratory experiments, available charts were examined on the original 66 sera. Charts were available from 33 of the original 66 sera. Most of the sera for which charts were unavailable were from unhospitalized patients and/or patients whose sera was sent to the Yale Rheumatology Laboratory from other physicians or laboratories in the community. The underlying disease processes in select patients are demonstrated in Tables 4, 5, and 6.

DISCUSSION

Serological tests are important tools in the diagnoses of many clinical diseases. Often, as for APS and SLE, they can support a diagnosis that is suspected clinically. In addition, certain serological markers can predict clinical course, assess risk from disease, or monitor disease progress. For instance, anti-dsDNA antibodies by Crithidia are regarded as very specific serological markers in confirming the diagnosis of SLE; in addition, the presence of anti-dsDNA antibodies detected by Crithidia can correlate with the activity of lupus nephritis. Because serological tests are often relied upon to diagnose disorders, it is essential that they are interpreted correctly and limitations of tests are acknowledged.

We assessed the specificity of serologic tests for SLE in the presence of ACA, as the latter autoantibody can be found in patients with SLE. A total of 66 sera were used for this study. Before proceeding with the adsorption of ACA, the 66 sera were re-evaluated for ACA reactivity at the beginning of the experiment. Eight sera were negative for ACA in our hands, suggesting some technical variability with the current tests and those previously performed.

Many sera had been stored for several years and it is possible that antibody denaturation could have contributed to the 8 negative ACA results, especially considering that the number of times that the sera had been thawed and frozen prior to this project was unknown. In most sera, however, the differences between the ACA IgG/Control ratios were not statistically significant, so the ACA ELISA technique used for this experiment was deemed acceptable and comparable to that of the Yale Rheumatology Diagnostic Laboratory.

After ACA adsorption, differences were noted in ANA, dsDNA and histone testing. Five ANA positive sera (Specimens 22, 25, 33, 42, 53) became negative after ACA adsorption. In 4 out of 5 of these patients, the ANA reactivity prior to adsorption was extremely low-titer at 1:40 and in the experience of the Yale Rheumatology Laboratory generally of no clinical importance. Seven sera (Specimens 1, 10, 50, 56, 57, and 62) demonstrated a change in ANA pattern following ACA adsorption; in all cases, the pattern changed from either nucleolar or speckled into a homogeneous pattern. Although it is tempting to speculate that the ACA could contribute to nucleolar or speckled staining patterns in some circumstances, there was no obvious correlation with ACA titer or clinical diagnoses in these patient samples.

Differences in anti-dsDNA antibody reactivity as determined by Crithidia immunofluorescence before and after adsorption of ACA were more striking. Table 4 lists the results of anti-dsDNA antibodies before and after ACA adsorption in parallel with clinical diagnoses. Seven out of 10 of the sera with positive anti-dsDNA antibodies prior to ACA adsorption had available patient charts. They were reviewed only after all laboratory work was completed, ensuring that the sera used during the experiment was

obtained and tested in a blinded fashion. Careful attention was focused on the 7 charts to look for underlying diagnoses. Patients 46, 56, and 65 all had a clinical diagnosis of SLE, low titer ACA, and demonstrated no change in anti-dsDNA antibody reactivity after ACA adsorption. Patient 57, also with an underlying diagnosis that included SLE, demonstrated a change in titer of anti-dsDNA antibodies after ACA adsorption, but was still positive for anti-dsDNA antibodies. This patient also had clinically apparent APS. Surprisingly, patients 12, 23, and 32—none of whom had an underlying diagnosis of SLE and were ANA negative—were anti-dsDNA antibody positive by Crithidia testing and lost this reactivity after ACA adsorption. Of particular note, patient 23 had high titer ACA (93 GPL Units) and high titer anti-dsDNA antibodies (>1:320), but following adsorption of ACA lost reactivity to anti-dsDNA antibodies.

The loss of reactivity for Crithidia anti-dsDNA antibodies in patients 12, 23, and 32 following ACA adsorption (all of whom had negative ANAs and no history of SLE from chart review) suggests that the reactivity to dsDNA might have been falsely positive due to the presence of ACA. This is intriguing given that the Crithidia test is considered the most specific test for anti-dsDNA antibodies; the dsDNA antibodies bind to the dsDNA kinetoplast present in the Crithidia organism. It is possible that in the ACA positive sera, immobilization of $ß_2$-glycoprotein I by an antigen within the kinetoplast contributed to a positive interpretation of the Crithidia anti-dsDNA antibody assay.

On the other hand, there was no loss of dsDNA reactivity for patients 46, 56, 57 and 65. These patients all had an underlying diagnosis of SLE, suggesting that true autoantibodies to dsDNA are not affected by ACA adsorption.

In contrast to anti-dsDNA tests, there were no significant differences in anti-histone reactivity before and after adsorption of ACA. Although 4 of 19 patient sera lost reactivity to anti-histone after ACA adsorption, a more accurate comparison using the anti-histone units/ml showed no significant changes before and after ACA adsorption. Two of the four patients that lost reactivity to anti-histone antibodies did not have SLE, and one had SLE with known APS. Thus, although the numbers are again low, the presence of ACA could contribute to anti-histone antibody positivity.

Specificity of serological tests, including the Crithidia immunofluorescence and ELISA tests for antibodies to dsDNA, depends on the interactions between antibodies and the antigen driving their production. Several factors can influence the binding of antibodies that cross-react or nonspecifically bind to antigens in the assay. Antibodies bind to antigens through specific amino acid residues which in part give the antigen a characteristic charge and configuration. The noncovalent binding between antibody and antigen consists of a combination of hydrophobic forces, Van der Waals forces, hydrogen bonds, electrostatic bonds, and dipole interactions. The strength of binding is further influenced by the three-dimensional fit between the antigen and the antigen binding site on the antibody. Although the strongest binding occurs when shapes match exactly (which occurs with somatically mutated, high affinity antibodies), antigens can bind to antibodies when shapes match less perfectly; however, the strength of binding is decreased. Thus, there are physical and chemical properties of antigens and antibodies that influence binding.[20,31]

In addition, there are general factors that can affect *in vitro* reactions between antigens and antibodies. The reaction conditions in the assays can

influence the strength of binding. The concentration of antigen and antibody, the pH and chemical composition of solutions, the size and charge of antigen and antibody, and the presence of other competing antibodies can all affect the noncovalent bond between antigen and antibody. Additionally, the three-dimensional fit between the antigen and antibody can be affected by reaction conditions. The antigen binding site on the antibody can undergo slight conformational change in order to accommodate the antigen. In an *in vitro* experiment, the structures of both antigen and antibody can be altered, thus affecting binding strength.[20,31] This is best exemplified by pathogenic ACAs, which bind $ß_2$-glycoprotein I. Plasma levels of $ß_2$-glycoprotein I are rarely affected by even high-titer ACA, as the conformation of $ß_2$-glycoprotein I in solution results in weak bonding affinity for ACA. In contrast, when plasma $ß_2$-glycoprotein I is immobilized by phospholipid bound to plastic (as in the ACA ELISA) or by activated endothelium, the binding affinity of ACA increases by several orders of magnitude. These factors should be considered when using serological tests to identify antibodies in sera from patients.

Weaknesses

There are several weaknesses in this study. First, the study was only a pilot study employing a relatively small sample size. A larger study in the future might reveal more or less significant results.

Although sera were initially aliquoted in triplicate in order to avoid repeated freezing and thawing, the effects of such cycles on reactivity to ACA, ANA, anti-dsDNA, and anti-histone antibodies cannot be determined. It is possible that freezing and thawing might have produced erroneous results. Furthermore, it is possible that the technique used for ACA adsorption might

have resulted in the inadvertent adsorption of other antibodies in addition to ACAs, which might account for the loss of reactivity to dsDNA.

The experiment addresses whether the presence of high-titer ACA can yield false positive anti-dsDNA antibodies. However, it does not attempt to answer the converse, i.e. whether the presence of high-titer anti-dsDNA antibodies can give rise to a false positive ACA.

While charts were obtained from patients on 50% of the sera, there were still 50% of sera for which no patient charts were available. It is difficult to determine precisely how the information from these patients might have affected the outcome of the experiment.

CONCLUSION

The newly emerging clinical spectrum of APS and its known association with SLE has made routine the screening of patients for both disorders. Our study suggests that some serologic tests, specifically the Crithidia luciliae immunofluorescence assay for anti-dsDNA antibodies, can be falsely positive in the presence of ACA. This finding underscores the need to interpret serologic tests in the context of the clinical situation.

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